

# Assessing cisplatin-induced ototoxicity and otoprotection in whole organ culture of the mouse inner ear in simulated microgravity



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## ABSTRACT

Cisplatin is a widely used anti-cancer drug. Ototoxicity is a major dose-limiting side-effect. A reproducible mammalian *in-vitro* model of cisplatin ototoxicity is required to screen and validate otoprotective drug candidates. We utilized a whole organ culture system of the postnatal mouse inner ear in a rotating wall vessel bioreactor under “simulated microgravity” culture conditions. As previously described this system allows whole organ culture of the inner ear and quantitative assessment of ototoxic effects of aminoglycoside induced hair cell loss. Here we demonstrate that this model is also applicable to the assessment of cisplatin induced ototoxicity. In this model cisplatin induced hair cell loss was dose and time dependent. Increasing exposure time of cisplatin led to decreasing EC<sub>50</sub> concentrations. Outer hair cells were more susceptible than inner hair cells, and hair cells in the cochlear base were more susceptible than hair cells in the cochlear apex. Initial cisplatin dose determined the final extent of hair cell loss irrespective if the drug was withdrawn or continued. Dose dependant otoprotection was demonstrated by co-administration of the antioxidant agent N-acetyl L-cysteine. The results support the use of this inner ear organ culture system as an *in vitro* assay and validation platform for inner ear toxicology and the search for otoprotective compounds.

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## 1. Introduction

In a recent survey of the International Agency for Research on Cancer 3.2 million cases of cancer were newly diagnosed in the European countries (Ferlay et al., 2010). In the treatment of cancer, cisplatin [cis-Diammin dichloroplatinum (II)] has remained an effective and widely used anti-cancer drug since it was first introduced to the clinic in the early 1970s. Applications include

a widespread range of cancers in children and adults. Adverse side-effects of cisplatin include nephrotoxicity, bone marrow toxicity, gastrointestinal toxicity, liver toxicity and neurotoxicity (Ekborn et al., 2003). Beside these, one of the major dose-limiting side effects is ototoxicity, often resulting in irreversible sensorineural hearing loss. For example, 20% of testicular cancer patients treated at standard dose experience persistent ototoxicity but it may affect more than 50% of patients receiving cumulative high doses of cisplatin > 400 mg (Bokemeyer et al., 1998). Up to 60% of paediatric cancer patients treated with cisplatin develop permanent bilateral hearing loss (Knight et al., 2005, 2007; Brock et al., 2012). In children, young age, male gender and increasing cumulative dose appear as critical risk factors for cisplatin induced ototoxicity (Yancey et al., 2012).

Cisplatin induced ototoxicity in the mammalian inner ear has been described and modelled in numerous animal studies (reviewed in: Rybak et al., 2007, 2009, 2012; Rybak and Ramkumar, 2007; Rybak and Whitworth, 2005; Schacht et al., 2012). In the cochlea cisplatin causes degeneration of three different types of cells; the hair cells in the organ of Corti (Anniko and Sobin, 1986; Fleischman et al., 1975; Zheng and Gao, 1996) the epithelial cells of

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the stria vascularis (Meech et al., 1998; Ravi et al., 1995) and neurons of the spiral ganglion (van Ruyven et al., 2005; Zheng and Gao, 1996; Zheng et al., 1995). A more profound toxic effect of cisplatin on SGN than on hair cells was found in experiments with cochlear explant cultures (Zheng and Gao, 1996). It is generally accepted that cisplatin ototoxicity in hair cells is mediated by reactive oxygen species (ROS) (Clerici et al., 1996; Dehne et al., 2001; Kim et al., 2010; Rybak, 2007; Rybak et al., 2009; Rybak and Ramkumar, 2007). Transcription factor NF- $\kappa$ B and high-mobility group (HMG) protein (Rybak and Ramkumar, 2007), transcription factor STAT1 (STAT; signal transducers and activators of transcription) (Kaur et al., 2011; Schmitt et al., 2009) and Nox3 (Rybak et al., 2012) are suggested as further death pathways. Currently, large scale drug screening for otoprotective agents in *in vivo* animal models is impeded by the mortality related to the systemic toxicity of cisplatin and the inter-individual variability of the observed ototoxic effects particularly in mice (Parham, 2011). Furthermore, the number of animals necessary to fully evaluate the number of potential ototoxic and otoprotective agents is prohibitive. Towards the development of an *in vivo* model a quantitative assay employing the non-mammalian model of the lateral line of zebrafish larvae has successfully been introduced to facilitate drug screening for ototoxic and otoprotective agents (Chiu et al., 2008; Hirose et al., 2011a; Ou et al., 2007; Ton and Parng, 2005). Hit compounds identified in the zebrafish screening system need to be validated in mammalian *in vitro* models.

Mammalian *in vitro* models to study cisplatin ototoxicity have utilized inner ear cell lines, tissue culture and to a certain extent whole organ culture. Immortalized cell lines have been generated from the auditory sensory epithelia of the H-2Kb-tsA58 transgenic mouse (Jat et al., 1991) at embryonic (Rivolta et al., 1998), postnatal (Kalinec et al., 2003) and functionally mature, adult-like (Kalinec et al., 1999) developmental stages. These cell lines have been employed for several purposes including the screening for toxic and protective agents (Kalinec et al., 2003; Rivolta and Holley, 2002). Tissue culture of isolated tissue fragments dissected from the postnatal inner ear has been used extensively for the study of cisplatin ototoxicity (Ding et al., 2011; Liu et al., 1998; Park et al., 2009; Previati et al., 2007; Yarin et al., 2005; Zhang et al., 2003). However, tissue culture models require micro-dissection of the auditory sensory epithelium which is technically demanding and time-consuming and puts a limit to its application as a validation tool for the increasing number of potential ototoxic and otoprotective agents that arise from the increasing number of screening efforts using inner ear derived cell lines or the zebrafish lateral line. Finally, organ culture models used for the study of cisplatin ototoxicity have been limited to developmental stages of the late embryonic inner ear (Anniko and Sobin, 1986).

In the present study we utilized our recently developed whole organ culture model of the postnatal inner ear in a rotating bioreactor culture system under “simulated microgravity” conditions (Arnold et al., 2010; Hahn et al., 2008). This system allows maintaining the entire mouse inner ear organ isolated at postnatal day 7 for up to seven days *in vitro* culture (Hahn et al., 2008). Here we demonstrate that in this model ototoxicity can be quantitatively assessed for cisplatin and also serve as a validation platform for otoprotective compounds such as the antioxidant agent N-acetyl L-cysteine (L-NAC). L-NAC is in clinical use for many years (review: Samuni et al., 2013). It is frequently prescribed as a mucolytic and antioxidative agent, due to its rapid reactions with oxygen and thyl radicals. L-NAC has recently (Riga et al., 2013; Yoo et al., 2013) been tested clinically in patients receiving cisplatin treatment. It was shown that L-NAC was at least a promising drug to prevent cisplatin-induced ototoxicity.

## 2. Material and methods

### 2.1. Animals

Mice used in this study were obtained from an in house breeding colony of either a 129SV (cisplatin ototoxicity) or NMRI (otoprotection) genetic background. Both mice strains gave similar results regarding cisplatin ototoxicity. One hundred fifty six animals were used in this study. Animal use for organ explantation was approved by the Committee for Animal Experiments of the Regional Council (Regierungspräsidium) of Tübingen (dated 19 October 2010 and 06 November 2012).

### 2.2. Organ culture system setup

Details of the methods were described previously (Arnold et al., 2010; Hahn et al., 2008). Briefly, inner ear organ explants were obtained from mice aged postnatal day 7 and 5–6.5 g body-weight. Whole organ inner ear explants were cultured in 55 ml High Aspect Ratio Vessel (HARV; Synthecon Inc., Houston, TX, USA) culture vessels mounted on a Rotary Cell Culture System (RCCS<sup>TM</sup>-4; Synthecon Inc., Houston, TX, USA). All dissections were carried out under sterile conditions in a laminar flow cell culture hood (HeraSafe KS12, Heraeus Instruments GmbH, Hanau, Germany). Mice pups at postnatal day 7 were decapitated. The complete inner ear bony labyrinth capsules were dissected from the skull base in ice-cold Hank's balanced saline solution (HBSS). After completion of the gross dissection, a micro-dissection opening the perilymphatic fluid spaces was carried out in order to provide access of the culture medium to the inner ear sensory epithelia. The bioreactor was placed in a 37 °C, humidified 5% CO<sub>2</sub>/95% air incubator (HeraCell CO2 Incubator, Heraeus Instruments GmbH, Hanau, Germany). Each incubator was installed with one RCCS<sup>TM</sup>-4 allowing the culture of up to four culture vessels in parallel in one incubator. Freshly dissected inner ear organ explants were inserted into the HARV culture vessels and suspended in cell culture medium. The HARV vessel was running at a rotation speed of 30 rounds/min (RPM). The culture medium was Neurobasal<sup>®</sup> Medium (Life Technologies<sup>TM</sup>, Darmstadt, Germany) supplemented with 1 × B27<sup>®</sup> supplement (Life Technologies<sup>TM</sup>, Gibco<sup>®</sup>, Darmstadt, Germany) 5 mM glutamine (Life Technologies<sup>TM</sup>, Invitrogen, Inc., Darmstadt, Germany), 10 mM Hepes (Life Technologies<sup>TM</sup>, Invitrogen, Inc., Darmstadt, Germany), and 100 U of penicillin (Sigma, St. Louis, MO, USA).

### 2.3. Application of cisplatin

Cisplatin (MW: 300.05 g/mol, Sigma–Aldrich, St. Louis, MO, USA; thus 1 µg/ml = 3.3 µM) was applied to the culture medium at different concentrations (up to 30 µg/ml, corresponding to approx. 99 µM) for 24, 48 or 96 h. In detail, concentrations used for 24 h were 1, 2.5, 5, 10, 15 and 30 µg/ml (corresponding molar concentrations: 3.3, 8.25, 16.5, 33, 49.5 and 99 µM), for 48 h 0.1, 0.3, 0.5, 1, 1.5, 1.75, 2, 2.5, 3 and 5 µg/ml (corresponding molar concentrations: 0.33, 0.99, 1.65, 3.3, 4.95, 5.77, 6.6, 8.25, 9.9 and 16.5 µM) and for 96 h 0.1, 0.3, 0.5, 1 and 2 µg/ml (corresponding molar concentrations: 0.33, 0.99, 1.65, 3.3, and 6.6 µM).

### 2.4. Application of L-NAC

The otoprotective compound L-NAC (N-acetyl-L-cysteine; Sigma–Aldrich, St. Louis, MO, USA) (Feghali et al., 2001; Ton and Parng, 2005) was applied at a concentration of 0.1, 0.3, 1 and 3 mM. For this series of experiments separate controls were made without

and with Cisplatin at 1.75 µg/ml, the EC<sub>50</sub> for OHC determined in the above described experiments.

### 2.5. Visualization of cochlear hair cells

After termination of the culture period, inner ear whole organ explants were fixed with phosphate-buffered formaldehyde solution 4% (Roti®-Histofix 4%, Karlsruhe, Germany). The organ of Corti was micro-dissected as a whole mount preparation and divided into basal, middle, and apical segments. The surface morphology of the organ of Corti, in particular hair cell stereocilia bundles, were visualized by phalloidin labelling of F-actin and immunohistochemical labelling for myosin VIIa. For immunohistochemistry, after a blocking step with 1% BSA in 0.2% Triton PBS, whole mount preparations were incubated overnight at 4 °C with a primary antibody for myosin VIIa, 1:500 (rabbit polyclonal, No: PTS-25-6790; Axxora, Proteus Biosciences, Ramona CA, USA). After washing with 0.2% Triton/PBS, primary antibodies were detected using Alexa-conjugated secondary antibodies for 60 min at RT 1:400 (Alexa488 donkey anti rabbit, No.: A21206; Molecular Probes, Life Technologies™, Darmstadt, Germany). The whole mount preparations were counterstained and incubated with phalloidin conjugated to the dye Alexa568 (Life Technologies™, Darmstadt, Germany) for 20 min in the dark at room temperature. After washing with 0.2% Triton in PBS specimens were incubated in DAPI (Sigma–Aldrich, St. Louis, USA) diluted 1:100 in PBS for 20 min. After a washing step in PBS, the whole mount specimens were mounted using FluorSave™ (Calbiochem, Merck KGaA, Darmstadt, Germany).

### 2.6. Quantification of cochlear sensory hair cell maintenance and statistical analysis

Quantification of surviving and lost sensory hair cell was performed for control, ototoxic and otoprotective conditions. The effects of these experimental paradigms were quantified in the whole mount preparations of the organ of Corti. The whole mount preparations were analysed using a Zeiss Axioplan 2 epifluorescence microscope and AxioVision Imaging Software (Zeiss, Göttingen, Germany).

The inner hair cells (IHC) and outer hair cells (OHC) were evaluated separately. Length measurements (mm) along the longitudinal axis of the organ of Corti were made in the midline between the inner and outer hair cell area corresponding to the pillar cell region for the total of all segments. The level of hair cell preservation was defined as the percentage of the length in the corresponding segments with more than 90% hair cells preserved, separately for inner and outer hair cells. All values of length measurements are presented as mean ± SD. Differences between experimental groups were assessed using JMP (Version 9; SAS Institute, Cary, USA). Paired Student's 2-tailed *t*-test were used (\**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001 values were considered significant). SigmaPlot (Version 8, Systat Software Inc., San Jose, USA) was used to fit a typical dose–response curve (four parameter logistic curve) with a variable slope parameter to obtain a median effective concentration (EC<sub>50</sub>):

$$y = \min + \frac{\max - \min}{1 + 10^{(\log EC_{50} - x) \cdot \text{Hillslope}}} \quad (1)$$

min: bottom of the curve; max: top of the curve; *x*: cisplatin concentration; EC<sub>50</sub>: median effective concentration; Hillslope: characterizes the slope of the curve at its midpoint.

### 2.7. Quantification of utricle sensory hair cell maintenance

The surface morphology of the utricle, in particular hair cells, were visualized by phalloidin labelling of F-actin and

**Table 1**

Survival of inner (IHC) and outer (OHC) hair cells after 24, 48 and 96 h in culture. Level denotes the cisplatin concentration (µg/ml), *N* the number of organ explants. Mean values (Mean) and standard deviation (Std Dev) are given.

Level (µg/ml)	N	IHC		OHC	
		Mean	Std Dev	Mean	Std Dev
24 h Cisplatin					
0	12	1	0	1	0
1	3	1	0	0.96	0.06
1.75	9	1	0	1	0
2.5	5	0.84	0.11	0.58	0.26
5	5	0.72	0.14	0.37	0.15
10	5	0.45	0.11	0.06	0.08
15	3	0.48	0.21	0.04	0.08
30	2	0	0	0	0
Total	44				
48 h Cisplatin					
0	14	1	0	1	0
0.1	6	0.94	0.07	1	0
0.3	5	1	0	1	0
0.5	6	0.9	0.12	0.88	0.1
1	3	0.94	0.11	0.92	0.13
1.25	6	0.76	0.16	0.75	0.14
1.5	9	0.7	0.09	0.47	0.17
1.75	9	0.44	0.17	0.33	0.1
2	6	0.42	0.16	0.13	0.09
2.5	2	0	0	0	0
3	3	0	0	0	0
5	3	0	0	0	0
Total	72				
96 h Cisplatin					
0	15	0.64	0.21	0.83	0.11
0.03	3	0.39	0.05	0.85	0.15
0.1	11	0.63	0.17	0.86	0.15
0.3	3	0.43	0.36	0.41	0.16
0.5	7	0.38	0.07	0.61	0.24
1	6	0.34	0.2	0.09	0.07
2	6	0.04	0.07	0	0
Total	51				

immunohistochemical labelling for myosin VIIa. Immunohistochemistry was performed similarly as for the cochlea. Hair cells were counted using ImageJ (Schneider et al., 2012) and the ITCN Plugin (Image-based Tool for Counting Nuclei, <http://www.bioimage.ucsb.edu/>).

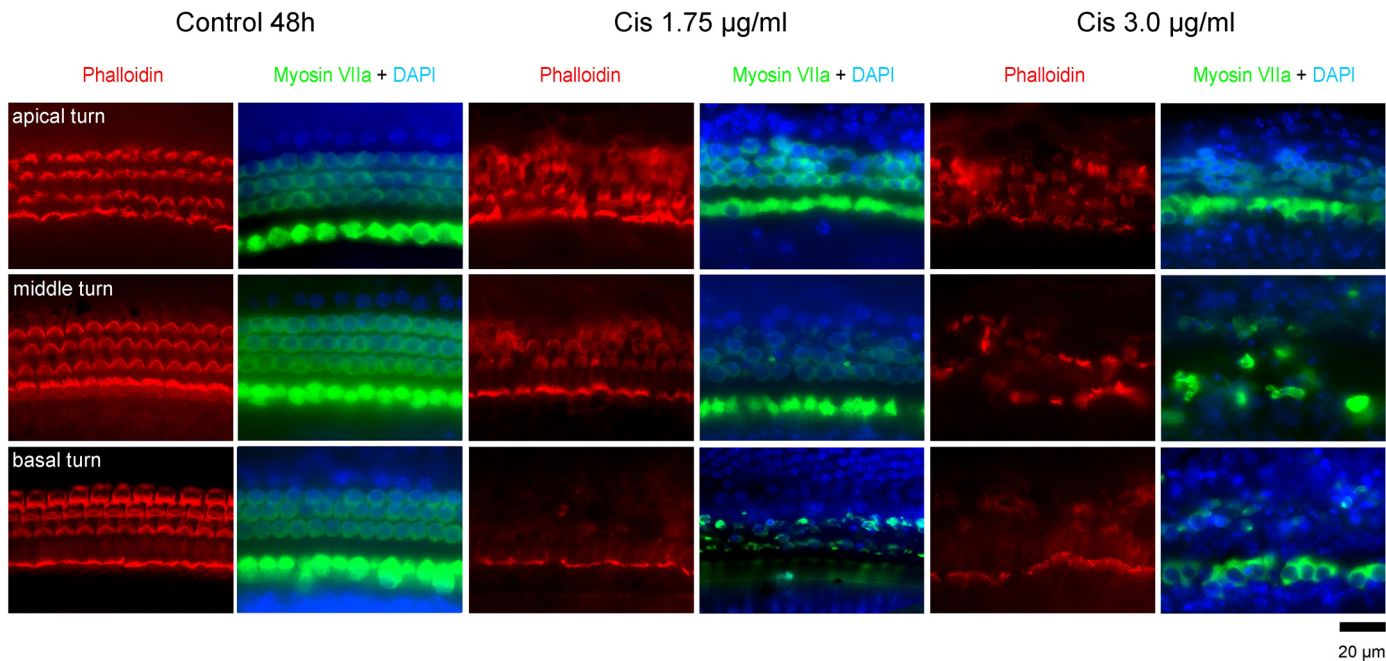
## 3. Results

### 3.1. General morphology

Hair cell damage and hair cell loss were observed in whole organ inner ear explants under simulated microgravity culture conditions after exposure to cisplatin for 24, 48 and 96 h. The concentration-dependent effect of cisplatin on hair cells was compared to control cultures without cisplatin exposure. Evaluation of the surface morphology of the organ of Corti using F-actin staining and immunohistochemical labelling with the hair cell marker myosin VIIa revealed a basal to apical gradient of hair cell loss (Fig. 1). Increasing cisplatin concentrations resulted in the progression of hair cell loss in a longitudinal gradient from the cochlear base towards the cochlear apex and in a radial gradient from outer hair cells to inner hair cells.

### 3.2. Dose response curves for cisplatin induced hair cell loss

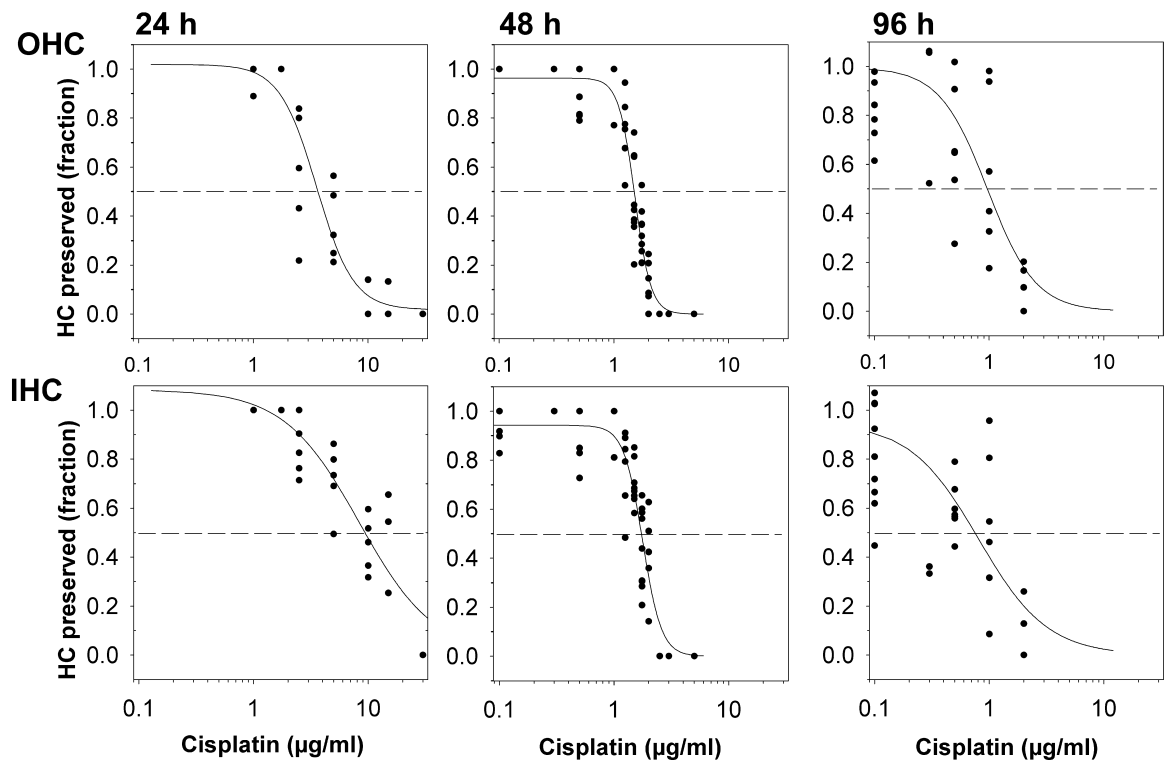
Effects of cisplatin exposure in microgravity culture were quantified using whole mount preparations of the organ of Corti. Control groups were compared to cisplatin exposure groups with different concentrations and durations of exposure. Table 1 shows the



**Fig. 1.** Photomicrographs of whole mounts of the apical, middle and basal turn (rows) of the cochlea of postnatal day 7 mice after 48 h in microgravity culture conditions. Preservation of hair cells is shown by F-actin staining with fluorescence-labelled phalloidin (red) and Myosin VIIa F-actin staining (green). Inner ears were treated with different concentrations (0, 1.75 and 3 µg/ml) of cisplatin (columns). In the control specimens complete hair cell preservation was observed. The base (lower row) to apex (upper row) gradient in hair cell damage was visible in the inner ears treated with 1.75 µg/ml cisplatin, hair cells in the base being completely wiped out and fully preserved in the apex. At 3 µg/ml cisplatin almost complete hair cell loss is visible. Bar = 20 µm.

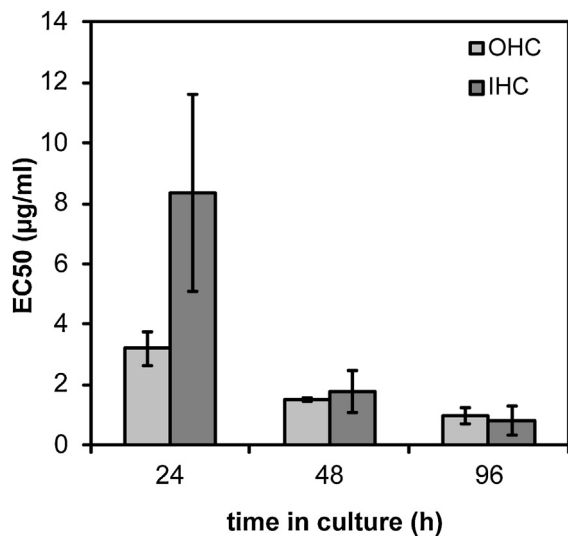
number of experiments and quantification of hair cell preservation (mean ± SDs) for the different groups. Calculations of hair cell preservation revealed that the loss of outer hair cells was statistically significant ( $p < 0.05$ ) for all cisplatin concentrations above

2.5 µg/ml and an exposure time of 24 h, above 1.25 µg/ml and an exposure time of 48 h and above 0.5 µg/ml and an exposure time of 96 h as compared to the untreated controls. Inner hair cell loss was statistically significant ( $p < 0.05$ ) for all cisplatin concentrations



**Fig. 2.** Dose–response curves calculated for outer (OHC, upper row) and inner hair cells (IHC, lower row) and different culture durations (left: 24 h, middle: 48 h, right: 96 h). Individual data points were normalized to the control data obtained without cisplatin in the culture medium (data not shown). Function regression lines were obtained using nonlinear regression analysis to fit a typical dose–response curve (Eq. (1)). The dotted line indicates 50% hair cell preservation.





**Fig. 3.** EC<sub>50</sub> values calculated from the function regression lines in Fig. 2 for inner (IHC) and outer hair cells (OHC) and at different culture durations (left: 24 h, middle: 48 h, right: 96 h). Doubling of exposure time approximately halved the EC<sub>50</sub> in the OHC. Vertical lines represent the standard error of the EC<sub>50</sub> values.

above 5 µg/ml and an exposure time of 24 h, above 1.25 µg/ml and an exposure time of 48 h and above 0.1 µg/ml and an exposure time of 96 h compared to the untreated controls. Complete outer hair cell loss was found at a concentration of 10 µg/ml and an exposure time of 24 h while complete inner hair cell loss was achieved at 30 µg/ml and an exposure time of 24 h. Much lower concentrations at and above 2.5 µg/ml were needed to induce complete outer and inner hair cell loss at an exposure time of 48 h. After 96 h of cisplatin exposure complete inner and outer hair cell loss was observed at and above a concentration of 2.0 µg/ml. At this prolonged culture duration some spontaneous loss of IHCs (preservation 64%; SD 21%) and OHCs (preservation 83%, SD 11%) was also observed in the basal segment under control conditions without cisplatin (Table 1).

Dose–response curves were calculated for inner and outer hair cells and different exposure times; loss of hair cells was normalized to the control data obtained without cisplatin (Fig. 2). For the 24 h exposure time EC<sub>50</sub> values were calculated as 8.34 µg/ml for the inner hair cells and 3.20 µg/ml for the outer hair cells. In the 48 h exposure group EC<sub>50</sub> values were reduced to 1.77 µg/ml for the inner hair cells and to 1.52 µg/ml for the outer hair cells. In the 96 h exposure group EC<sub>50</sub> values were further reduced to 0.80 µg/ml for the inner hair cells, and 0.97 µg/ml for the outer hair cells. For the outer hair cells, doubling the exposure time resulted in approximately halve the EC<sub>50</sub>, for the inner hair cells doubling the exposure time resulted in approximately a quarter the EC<sub>50</sub> (Fig. 3).

We examined the utricles of ears exposed 48 h to a dose of 1.75 µg/ml cisplatin and unexposed negative controls (Supplemental Fig. 1A and B). Hair cell counts were in the order of previous reports (Li et al., 2008) and revealed no significant ( $t$ -test:  $p = 0.93$ ) hair cell loss with this exposure parameter. In control ( $n = 6$ ) conditions  $3040 \pm 634$  hair cells were counted, after exposure to 1.75 µg/ml Cisplatin ( $n = 5$ )  $3069 \pm 421$  hair cells (Supplemental Fig. 1C). This is not unexpected for 1.75 µg/ml Cisplatin as significant hair cell loss was previously (Cunningham and Brandon, 2006) only seen at a more than 10 fold concentration (20 and 25 µg/ml cisplatin).

### 3.3. Culture duration and cisplatin dose

To test if the reduction of the EC<sub>50</sub> was predominantly a dose- or duration-dependent effect, in an additional set of experiments

cisplatin was added to the medium for an initial culture period of 24 or 48 h and was then removed (Fig. 4). These explants were continued in culture for a subsequent culture period without cisplatin for an additional 72 or 48 h, respectively. In both cases the total culture time summed up to 96 h. Cisplatin concentrations used for these experiments were 0.5 and 1 µg/ml and were based on the EC<sub>50</sub> values generated with the 96 h exposure time as described above. After initial culture periods of 24 and 48 h only a minor hair cell loss was observed. However, a progressive loss of hair cells was seen after completion of the 96 h total culture time despite the removal of cisplatin from the culture medium during the second time intervals. Surprisingly, no difference was seen between samples that had been exposed to cisplatin for the entire culture period of 96 h using the same concentrations. Obviously, the initial cisplatin concentration appeared to determine the degree of hair cell loss irrespective of the duration of the exposure in this setting.

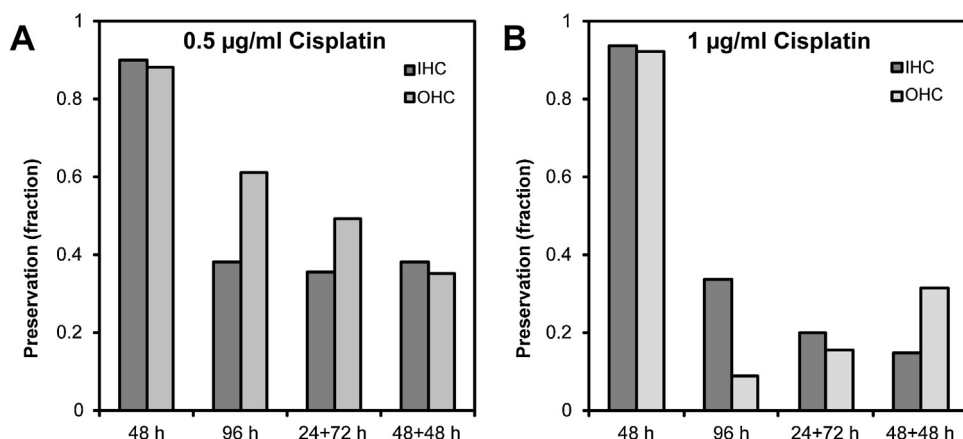
### 3.4. Otoprotection

In order to validate the use of the culture system for the evaluation of otoprotective compounds the otoprotective agent N-acetyl L-cysteine (L-NAC, at 0.1, 0.3, 1 ( $n = 17$  each) and 3 mM,  $n = 12$ ) was co-administered with cisplatin (1.75 µg/ml) for 48 h (Fig. 5) in an additional set of experiments. A dose–response curve was calculated between 0.1 and 3 mM L-NAC. In this set of experiments OHC preservation was 92% ( $n = 25$ , SD: 18%) in the untreated explants (0 µg/ml Cisplatin, 0 mM L-NAC). In the 1.75 µg/ml cisplatin treated explants OHC preservation dropped to 45% ( $n = 25$ ; SD 25%). These results correspond to the values determined in the first set of experiments. In 1.75 µg/ml cisplatin plus 1 or 3 mM L-NAC co-treated explants OHC preservation was quantified as 80% (SD: 20%) and 88% (SD: 22%) respectively, which is a highly significant ( $T$ -test:  $p < 0.001$ ) preservation of outer hair cells when compared to the 1.75 µg/ml cisplatin treated cultures. Co-treatment of 1.75 µg/ml cisplatin with 0.1 or 0.3 mM L-NAC did not result in a significant rescue of OHC. Under these experimental conditions the EC<sub>50</sub> for L-NAC was determined to be  $0.70 (\pm 0.29)$  mM for the OHC.

As expected from the first set of experiments, treatment with 1.75 µg/ml Cisplatin resulted in IHC-preservation being higher (81%, SD: 19%) than in OHCs. A rescue effect to 90% was nevertheless also seen in the treatments with 1 or 3 mM, though not significant.

## 4. Discussion

The present observations extend our previous studies (Arnold et al., 2010; Hahn et al., 2008) of a whole organ culture system of the inner ear using a rotating bioreactor in “simulated microgravity” to cisplatin ototoxicity and otoprotection. We demonstrate that hair cell loss can be induced by cisplatin in a dose-dependent fashion. Furthermore, hair cell loss can be prevented by the use of an otoprotective agent. The findings show that outer hair cells are more susceptible than inner hair cells, and hair cells in the base of the cochlea are more susceptible than hair cells in the apex. It is a general observation that the base of the cochlea is more vulnerable to traumatic insults than the apex as seen in the pattern of hair cell damage by cisplatin or aminoglycosides (Richardson and Russell, 1991; Rybak, 2007). In the organ culture system employed here this differential susceptibility is maintained. This suggests an intrinsic difference in sensitivity of the hair cells to cisplatin along a basal to apical gradient as the complete perilymphatic space is accessible to the drug. This effect may be explained by lower levels of the antioxidant glutathione in the cochlear base, which might result in the accelerated death of basal outer hair cells due to free-radicals (Sha et al., 2001). This is in agreement with an intrinsic susceptibility to free radicals that differs among cochlear cell populations.

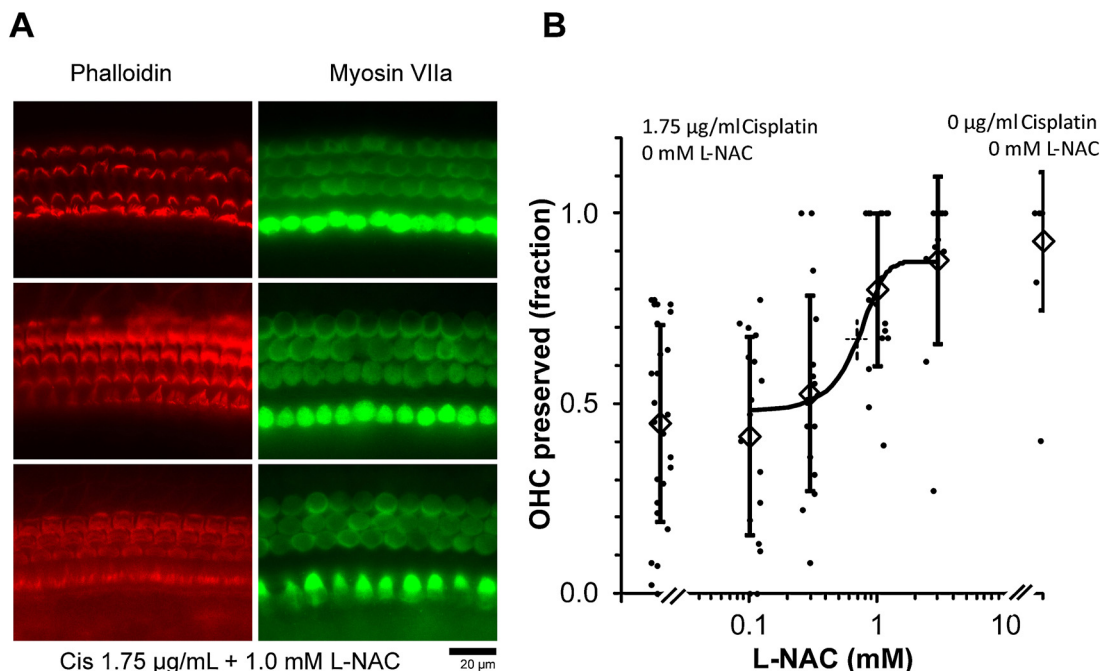


**Fig. 4.** Preservation of inner (IHC) and outer hair cells (OHC) at 0.5 µg/ml (left) and 1 µg/ml (right) cisplatin and different combinations of cisplatin exposure time and culture duration. Using 0.5 µg/ml (A) and 1 µg/ml (B) cisplatin at a culture duration of 48 h hair cell preservation was almost complete. Extending culture duration to 96 h resulted in a marked reduction of preserved hair cells. This reduction remained constant, regardless of the removal of the cisplatin after 24 or 48 h for the remaining culture duration (72 and 48 h respectively).

Several mammalian models have been devised and applied to the study of cisplatin ototoxicity. These models include cell culture models using cell lines derived from the organ of Corti, tissue culture dissected from the postnatal inner ear and organ cultures from embryonic stages. The conditionally immortalized organ of Corti derived cell lines UB/OC-1 and UB/OC-2 (Rivolta et al., 1998), OC-k3 (Kalinec et al., 1999) and HEI-OC1 (Kalinec et al., 2003) were established from the H-2Kb-tsA58 (Immortomouse) transgenic mice (Jat et al., 1991).

The UB/OC-1 cells were used to study cisplatin induced ototoxicity and otoprotection (Kaur et al., 2011; Mukherjee et al., 2010;

Mukherjee et al., 2011; Mukherjee et al., 2008), recently reviewed by Rybak et al. (2012). In these experiments using the UB/OC-1 cell line a cisplatin concentration of 2.5–10 µM (0.8–3.3 µg/ml) was used. The OC-k3 cell line was also used to study cisplatin induced ototoxicity (Bertolaso et al., 2001; Low et al., 2010; Previati et al., 2007; Previati et al., 2004). Cisplatin concentrations that effectively induced cell death in the OC-k3 cell line after 48 h incubation ranged from 1.7 µM (0.5 µg/ml) (Low et al., 2010) to 50 µM (15 µg/ml). The HEI-OC1 cell line was the most extensively utilized cell line in cisplatin induced ototoxicity and otoprotection experiments (Altun et al., 2014; Chen et al., 2012; Choi et al., 2011a,b; Im et al., 2010;



**Fig. 5.** Protection of outer hair cells (OHC) treated with cisplatin (1.75 µg/ml) and L-NAC (1 mM) in the inner ear culture system. (A) Photomicrographs of whole mounts of the apical, middle and basal turn (rows) of the cochlea of postnatal day 7 mice after 48 h in microgravity culture conditions. Preservation of hair cells is shown by F-actin staining with fluorescence-labelled phalloidin (red) and Myosin VIIa F-actin staining (green). The comparison to the middle panel of Fig. 1 exemplifies the well preserved hair cell structure due to the addition of L-NAC. (B) Dose response curve for otoprotective effects of L-NAC at 0.1, 0.3, 1 and 3 mM concentrations on outer hair cell (OHC) loss induced by cisplatin (1.75 µg/ml). Small effects were seen at 0.3 mM L-NAC, while adding L-NAC at 3 mM resulted in complete protection of the outer hair cells. Mean values of the different experimental conditions are given by diamonds, vertical lines represent one standard deviation. For better visibility of experimental results (full dots), values on the x-axis are randomly scattered around the respective value of L-NAC concentrations. Negative control conditions with 1.75 µg/ml Cisplatin without L-NAC are shown to the left; positive control conditions without Cisplatin and without L-NAC are shown to the right. The broken lines indicate the EC<sub>50</sub> value for L-NAC as related to the 1.75 µg/ml Cisplatin treatment. The continuous curve shows the nonlinear function regression line of a logistic function type (Eq. (1)).

Jeong et al., 2011; Kim et al., 2006, 2009, 2010, 2011; Lee et al., 2011; Myung et al., 2011; Oh et al., 2011; Park et al., 2012; So et al., 2008; Yu et al., 2010; Shin et al., 2012). In these studies cisplatin concentrations of 10–20  $\mu\text{M}$  (–3 to 6  $\mu\text{g/ml}$ ) led to cell death of about 50% of the cells after exposure times to cisplatin ranging from 24 to 72 h. However, quantitative data concerning dose– (or time–) response data can be extracted from only a limited number of reports: 50% viability was reported after 21 h incubation using 20  $\mu\text{M}$  (6  $\mu\text{g/ml}$ ) cisplatin (Kim et al., 2010); 50% viability was reported after 55 h incubation using 20  $\mu\text{M}$  (6  $\mu\text{g/ml}$ ) and after 36 h incubation using 40  $\mu\text{M}$  (13  $\mu\text{g/ml}$ ) (Im et al., 2010). An  $\text{EC}_{50}$  of 3.3  $\mu\text{M}$  (1  $\mu\text{g/ml}$ ) at 24 h exposure to cisplatin can be extracted from a dose–response curve in a recent report (Lee et al., 2011). This latter value is about an order of magnitude lower when compared to the previous reports (Im et al., 2010; Kim et al., 2010). In summary, after 24 h cisplatin incubation the 50% cell viability data from HEI-OC1 cell line reported are in the range of 3–20  $\mu\text{M}$  (1–6  $\mu\text{g/ml}$ ).

Tissue cultures prepared from the postnatal rat inner ear were also applied to study cisplatin ototoxicity. In these rat tissue culture studies cisplatin concentrations had a similar range as the concentrations used in the organ culture system of the present study (Cheng et al., 1999; Coling et al., 2007; Du et al., 2011; Kim et al., 2009; Kopke et al., 1997; Liu et al., 1998; Park et al., 2009; Yarin et al., 2005; Zhang et al., 2003; Zheng and Gao, 1996). In summary, in these studies a hair cell loss of 50% was observed at cisplatin concentrations of 15–30  $\mu\text{M}$  (5–10  $\mu\text{g/ml}$ ) at an exposure time of 24 h (see Supplemental Table 1 for details). In summary, after 24 h cisplatin incubation the 50% cell viability data from tissue culture experiments from the rat organ of Corti reported are in the range of 16–33  $\mu\text{M}$  (5–10  $\mu\text{g/ml}$ ).

In experiments performed *in vivo*, different regimes with either single or multiple cisplatin applications were used (Supplemental Table 2). In guinea pigs the dosages in single applications ranged from 5 to 20 mg/kg (Fleischman et al., 1975; Kopecky et al., 2012; Lee et al., 2011; Xiong et al., 2011; Yin et al., 2009) with significant hearing loss and hair cell deletion at dosages of 10 mg/kg (Daldal et al., 2007; Murphy and Daniel, 2011; Ramirez-Camacho et al., 2008; Wang et al., 2004; Yin et al., 2009) and above. Dosages above 14 mg/kg showed an increasing lethality. In guinea pigs the ototoxic dosages in multiple applications start from 1.5 mg/kg/day (Sepmeijer and Klis, 2009; Stengs et al., 1998) for 6 up to 22 consecutive days (until hearing loss was observed). Higher dosages were also used, 3 mg/kg/week for up to 8 weeks (Nader et al., 2010; Saliba et al., 2010) and up to 10 mg/kg/day for 2 days (Choe et al., 2004). In female Fisher344 rats two, four-day cycles of cisplatin (2 mg/kg), separated by a 10-day rest were applied to induce an ototoxic effect (Guthrie et al., 2008), resulting in a moderate hearing loss of 15 dB. A single dose of 16 mg/kg resulted in a more severe hearing loss of up to 70 dB and complete OHC loss in the cochlear base (Campbell et al., 1996; Kamimura et al., 1999). Single doses of 11–13 mg/kg cisplatin resulted in 20–40 dB hearing loss and a 50–60% loss or damage of hair cells (Kapusuz et al., 2013; Kaur et al., 2011; Mukherjee et al., 2010; Mukherjee et al., 2008; Mukherjee et al., 2006). In a long term study 7 mg/kg led to an additive interaction between cisplatin ototoxicity and age-related hearing loss (Bielefeld, 2013).

Compared to rat and guinea pig (Adams, 2002) the inner ear of mice appears the least sensitive to cisplatin *in vivo*. Using a single dose of 16 mg/kg i.p. (Kim et al., 2009; Parham, 2011) or application of a four-day 4 mg/kg (per day) regime (Oh et al., 2011; Park et al., 2009) or 2 times 5 mg/kg (Oh et al., 2011) resulted in only moderate hearing loss of about 20 dB. Using a single dose of 20 mg/kg i.p. cisplatin (Kopecky et al., 2012), hearing loss up to 35 dB in the mid-frequency range was achieved. Using the same dose, high-frequency loss and outer hair cell loss was observed 72 h following treatment (Schmitt and Rubel, 2013). To conclude, as a rough estimate, an *in vivo* ototoxic effect was observed between 10

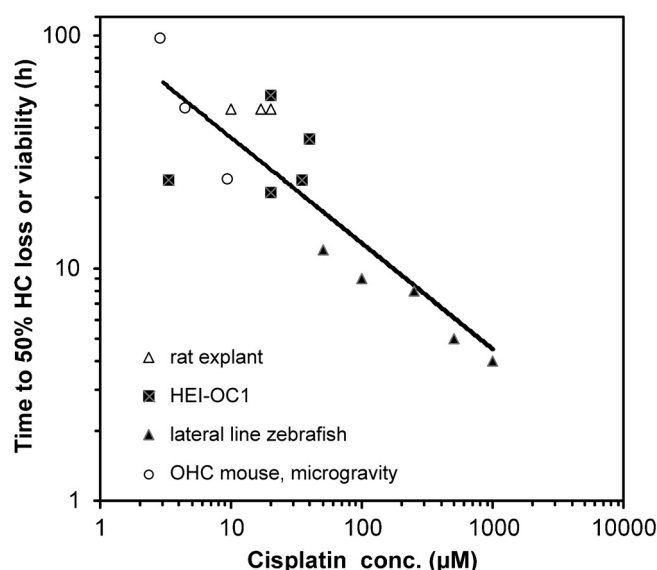
and 20 mg/kg either as a single or cumulative cisplatin dose. Assuming an equal distribution within the body, this corresponds to a dosage of 10–20  $\mu\text{g/ml}$  (approx. 30–60  $\mu\text{M}$ ), which is well within the range used *in vitro* studies utilizing cochlear derived cells. A recent *in vivo* pharmacokinetic study of cisplatin in the guinea pig demonstrated that a single dose of 8 mg/kg body weight of cisplatin applied intravenously resulted in an early high concentration of cisplatin in the base of the cochlea and a delayed elimination of cisplatin from the scala tympani perilymph compared to blood. At 1 h after the application the levels of cisplatin in perilymph and blood were equivalent at a concentration of approx. 6  $\mu\text{M}$  (corresponding to approx. 2  $\mu\text{g/ml}$ ) and further decreased thereafter (Hellberg et al., 2013). In summary, this result indicates that *in vivo* a single ototoxic dose of systemic cisplatin of 8 mg/kg results in perilymphatic cisplatin concentration of 6  $\mu\text{M}$  (2  $\mu\text{g/ml}$ ) in which is well in the range of mammalian *in vitro* preparations, as mentioned above are 3–20  $\mu\text{M}$  (1–6  $\mu\text{g/ml}$ ) for cell lines and 15–33  $\mu\text{M}$  (5–10  $\mu\text{g/ml}$ ) for tissue culture explants. In the organ culture system of the present study the ototoxic effect on the outer hair cells had an  $\text{EC}_{50}$  value of 11.5  $\mu\text{M}$  (3.50  $\mu\text{g/ml}$ ) at 24 h cisplatin exposure decreasing to 5  $\mu\text{M}$  (1.52  $\mu\text{g/ml}$ ) for 48 h exposure time, also appears close to the *in vivo* situation and other mammalian *in vitro* models.

Moving to the human situation and considering the long half-life of cisplatin in humans (long term elimination:  $327 \pm 91$  h (Monjanel-Mouterde et al., 2003), this may also roughly represent the concentration recommended for use in humans (50–120 mg/m<sup>2</sup> BSA; average BSA 1.9 m<sup>2</sup>  $\approx$  70 kg; 50 mg  $\cdot$  1.9 per 70 kg = 1.4 mg/kg; 120 mg  $\cdot$  1.9 per 70 kg = 3.3 mg/kg). Although varying considerably, average peak plasma concentrations between 1.7 up to roughly 10  $\mu\text{g/ml}$  (6–33  $\mu\text{M}$ ) were observed (Vermorken et al., 1986) after infusion of 100 mg/m<sup>2</sup> cisplatin. In patients receiving high dose cisplatin (150–225 mg/m<sup>2</sup>), 100% of patients showed hearing loss in the very high frequencies above 9 kHz (Kopelman et al., 1988). If an average of 70 kg body weight is assumed, this high dose cisplatin regime would correspond to 4–6 mg/kg which is in the range of ototoxic dosage in experimental animals (supplemental Table 2). A cumulative cisplatin dose of over 400 mg/m<sup>2</sup> resulted in hearing loss in more than 50% of patients (Bokemeyer et al., 1998). In a recent retrospective analysis of 102 pediatric patients 81.7% of the patients developed hearing loss after a mean cumulative cisplatin dose of 417.7 mg/m<sup>2</sup> (Yancey et al., 2012). In summary, the cisplatin concentrations obtained to be toxic to hair cells in the current study are well within the range of other *in vitro* and *in vivo* studies as well as the range of concentrations that are used in clinical applications.

A non-mammalian model to study cisplatin ototoxicity, a larvae zebrafish lateral line model, has been introduced (Ou et al., 2007; reviewed in (Brack and Ramcharitar, 2012; Buck et al., 2012; Coffin et al., 2010; Hirose et al., 2011b; Ou et al., 2010). The model shows evidence of dose-dependent cisplatin induced hair cell loss. The zebrafish larvae are available in large numbers and access to hair cells on the body surface lateral line allows screening in alive animals (Buck et al., 2012; (Shin et al., 2013; Shin et al., 2012). However, the cisplatin dosages applied to achieve hair cell loss are considerably higher (1–2 orders of magnitude; up to 1000  $\mu\text{M}$   $\approx$  300  $\mu\text{g/ml}$ ) than the cisplatin dosages required in the mammalian models mentioned above and the exposure times are considerably lower (in the range of several hours) than in mammalian *in vitro* and *in vivo* models (Buck et al., 2012; Hong et al., 2013; Ou et al., 2007).

Since biological effects of drugs may be different at low and high dosage, interpreting results from the zebrafish model may require some caution. It is argued (Ou et al., 2007) that the use of a much shorter exposure time in the zebrafish (several hours) require higher doses of cisplatin and simply result in an earlier initiation of a cell death cascade (Ou et al., 2007). Combining the





**Fig. 6.** Compiled data of 50% effect on hair cells in different experimental conditions as indicated in the legend. Regression analysis using a power law function ( $f(x) = ax^k$ ) on these data plotted on a log–log scale. The regression-analysis resulted in a straight line. This common nonlinear dependency between exposure time and  $EC_{50}$  across the species indicates a common mechanism in damaging hair cells in lateral line of zebrafish and hair cells in mouse. Data for HEI-OC1 were taken from: Chen et al., 2012; Im et al., 2010; Lee et al., 2011; Kim et al., 2010. Data for rat explants were taken from: Kopke et al., 1997; Zheng and Gao, 1996; Zhang et al., 2003. Data from zebrafish lateral line stem from Ou et al. (2007).

$EC_{50}$ -data on hair cell loss from the zebrafish (Ou et al., 2007) and the  $EC_{50}$ -data obtained in the present study on the loss of OHCs as well as representative studies from inner ear cell lines and tissue culture supports this hypothesis. A power law function ( $f(x) = ax^k$ ) is adequate to fit the combined data (Fig. 6). On a log–log plot the regression-line results in a straight line, where the slope of the line is equal to the power exponent  $k$  (because  $\log(f(x)) = k \cdot \log(x) + a$ ). This common nonlinear dependency between exposure time and  $EC_{50}$  across the species and methods indicates a common mechanism in damaging hair cells from the lateral line and the organ of Corti *in vitro* and *in vivo*. However, this does not exclude that different pathways are initiated in mammals and zebrafish; moreover in zebrafish the lowest toxic cisplatin concentration ( $50 \mu\text{M} = 15 \mu\text{g/ml}$ ) is still above the concentration used in most mammalian *in vitro* studies excluding a direct comparison under the same parameters. In this regard the different mechanism of apoptotic or necrotic cell death have to be considered. The primary biological target of cisplatin is DNA (reviewed in: Jamieson and Lippard, 1999; Langer et al., 2013; Wang and Lippard, 2004). Subsequently cell death may occur *via* several, complex signalling pathways (reviewed in Rybak et al., 2009). In a study on primary cultures of mouse proximal tubular cells apoptotic *versus* necrotic cell death is dependent on the cisplatin concentration (Lieberthal et al., 1996), high concentrations of cisplatin ( $800 \mu\text{M}$ ) led to necrotic cell death in an exposure time of 4 h while much lower concentrations of cisplatin ( $8 \mu\text{M}$ ) led to apoptosis over an exposure time of 4 days. Furthermore, this study concludes that reactive oxygen species play a role in mediating apoptosis but not necrosis. This notion derived from a kidney cell line is supported by the recent finding that HEI-OC1 auditory cells treated with  $20 \mu\text{M}$  ( $6 \mu\text{g/ml}$ ) cisplatin increased the production of intracellular ROS and cell death in a time-dependent manner of treatment (Chen et al., 2012; Kim et al., 2010). Considering further differences between the mammalian and the fish model is the lack of compartmentalization of inner ear fluids in fish. Also there are no outer and inner hair cells in the neuromast. Furthermore spontaneous regeneration of hair cells

in the lateral line occurs within 24–72 h (Mackenzie and Raible, 2012) prohibiting the 24–96 h time windows of exposure used in mammalian *in vitro* systems. Consequently results from zebrafish screening efforts need to be confirmed and validated in mammalian models.

In conclusion, the proposed whole organ model allows culturing the intact postnatal mammalian inner ear sufficiently long to observe relevant cell biological processes in cisplatin induced ototoxicity and its protection by an antioxidative agent. Pharmacological studies can be performed in dose ranges comparable to other mammalian *in vitro* and *in vivo* models and in the range of concentrations, which are used therapeutically in humans. Though performed *in vitro*, this whole organ ototoxicity and otoprotection assay of the postnatal ear preserve the structure of the organ and most closely resembles the *in vivo* situation.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2014.03.022>.

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